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Cell Permeability: a Factor in the Biotin-Oleate Relationship in *Lactobacillus arabinosus*

II. Effect of Oleic Acid and Other Surfactants on Free Biotin Uptake

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ABSTRACT

Bound biotin-saturated cells were incubated in the presence of biotin and glucose (37 C, *p*H 7.5) with or without oleic acid, Tween 20, 40, 60, and 80, Aerosol OT, sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide, Triton X-100, Non-Ion-Ox, and Haemo-Sol. With low concentrations (up to 5 μg/ml) and short reaction times (up to 10 min), oleic acid stimulated free biotin accumulation. Increased concentrations (10 to 50 μg/ml) or reaction times (10 to 30 min) caused progressive reductions in uptake or increased release of previously accumulated vitamin. Combination of Tween 40 (1 mg/ml) with oleic acid (up to 50 μg/ml) detoxified oleic acid and stimulated free biotin uptake. Oleic acid (5 μg/ml or more) reduced cell viability, an effect which was overcome by Tween 40. All other surfactants tested stimulated free biotin accumulation at sublethal concentrations. Aerosol OT and SDS exhibited the same degree of stimulatory activity as detoxified oleic acid; however, at concentrations higher than 200 μM, a rapid decrease in vitamin accumulation was observed which paralleled that caused by increased oleic acid concentrations. The results suggest that oleic acid and other surfactants affect the permeability of cells of *Lactobacillus plantarum* (formerly called *L. arabinosus*) in a similar manner.

Oleic acid and certain other fatty acids are known to exert a "sparing" effect on the requirement of certain lactic acid organisms for biotin (17), pantothenic acid (4), and riboflavine (13). Moreover, growth stimulation of *Lactobacillus casei* by oleic acid in pantothenate- and riboflavine-free media was eliminated by removing contaminating traces of these vitamins (12). The mechanism by which oleic acid spares the biotin requirement of certain organisms has not been elucidated fully.

It is the purpose of this report to show that oleic acid and other surface-active agents stimulate the uptake of biotin by cells of *L. plantarum*.

MATERIALS AND METHODS

L. plantarum strain 17-5 (ATCC 8014) (formerly called *L. arabinosus*) was used in all studies and also as the assay organism. The materials and methods were those described previously (15), except that the cells were grown in 5×10^{-5} μg of biotin per ml of growth medium, the cell concentration in the reaction mixtures was approximately 0.3 mg/ml in all cases, the reaction temperature and *p*H were 37 C and 7.5 (0.1 M PO_4), respectively, and the reaction mixtures contained 200×10^{-4} to 250×10^{-4} μg of biotin per ml, 0.5% NaCl, and 1% glucose. Bound biotin-saturated cells were prepared as described earlier (15). The surface-active agents tested were oleic acid (Calbiochem), sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB; Matheson, Coleman and Bell, East Rutherford, N.J.), sodium sulfosuccinate (Aerosol OT; Fischer Scientific Co., Pittsburgh, Pa.), Triton X-100 (Rohm & Haas Co., Philadelphia, Pa.), Non-Ion-Ox (Aloe Scientific, St. Louis, Mo.), Tweens 20, 40, 60, and 80 (polyoxyethylene sorbitan mono-laurate, -palmitate, -stearate, and -oleate; Nutritional Biochemical Corp., Cleveland, Ohio), and Haemo-Sol (Meineke Corp., Baltimore, Md.). All agents except oleic acid were dissolved in water. An aqueous emulsion of oleic acid (5.0 mg/ml) was prepared by mixing 50 mg of the fatty acid with 10 ml of distilled water in a Mini-mill (Gifford-Wood Co., Hudson, N.Y.) for 5 min. Suspensions so prepared remained stable for several hours. Fresh emulsions of oleic acid were prepared just prior to use.

RESULTS

Effect of oleic acid and Tweens on cell viability and free biotin uptake. The data presented in

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Fig. 1A show that viability decreased rapidly when cells were exposed to oleic acid, especially at concentrations above 5 μg/ml. Moreover, cell viability decreased with increased time of exposure to fatty acid. It is seen from Fig. 1B that short exposures of the cells to low concentrations of oleic acid caused significant increases in free biotin uptake (>55% increase at 6 min with 5 μg/ml). However, increasing concentrations of oleic acid caused a rapid decrease in uptake. It is also evident that increasing the time of contact between cells and free fatty acid resulted in progressive decreases in vitamin uptake, such that at 30 min a marked decrease was observed with 5 μg/ml. The decrease in uptake activity can be correlated directly with the decrease in cell viability. The data in Fig. 1B also demonstrate the expected detoxifying effect of Tween 40 on oleic acid. Concomitant with detoxification was a rapid and marked stimulation of free biotin uptake (Fig. 1B), exhibiting maximal effect between 5 and 25 μg of oleic acid per ml.

Since Tween 40 is a surface-active agent, its effect on biotin uptake both alone and in combination with oleic acid was tested. The data (Fig. 2)

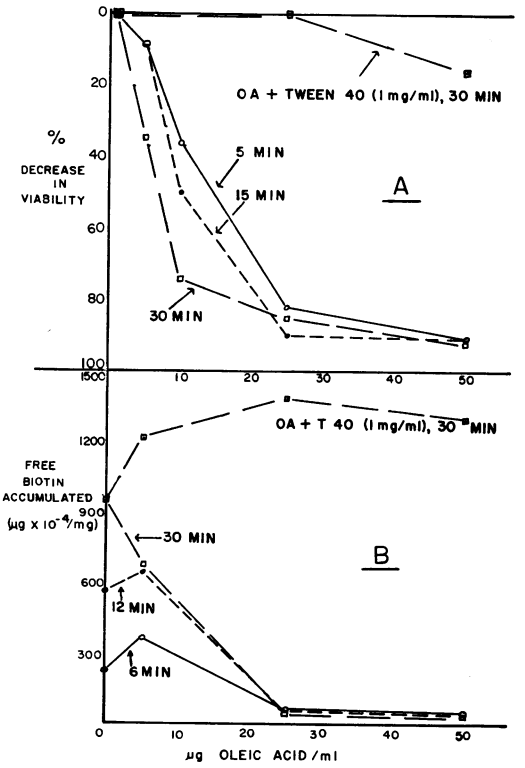

FIG. 1. *Effect of oleic acid and oleic acid plus Tween 40 on free biotin uptake and cell viability.*

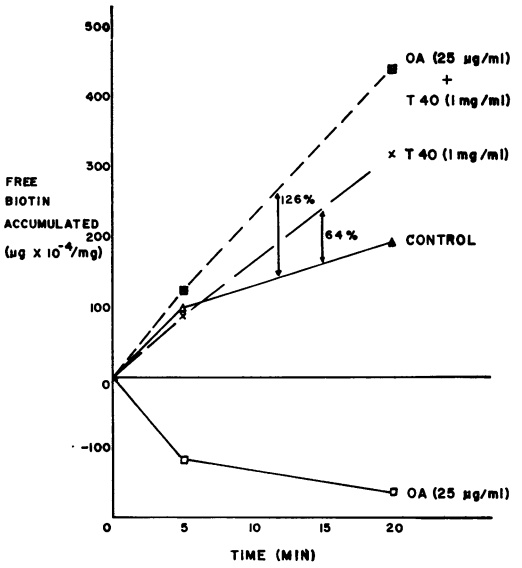

FIG. 2. *Effect of oleic acid, Tween 40, and oleic acid plus Tween 40 on free biotin uptake.*

show that Tween 40 alone (1 mg/ml) stimulated vitamin uptake by 64% in 20 min, whereas, in combination with oleic acid (25 μg/ml), an additive effect resulting in a 126% increase was observed. Oleic acid alone (25 μg/ml) caused a rapid decrease in internal free biotin, again demonstrating the detoxifying effect of Tween 40 on oleic acid as well as the toxicity of the free fatty acid for these bacterial cells.

Inasmuch as other Tweens also possess surface-active properties, their effects on the accumulation system were studied. The data in Table 1, experiment 1, show that all Tweens tested stimulated free biotin accumulation. Of interest is the fact that 25 mg of Tween 80 per ml caused some inhibition of uptake after 60 min of incubation. Since it has been shown (8) that 3% of the total fatty acid present in commercial preparations of Tween 80 is oleic acid, it was assumed that the Tween 80-induced inhibition was due to the presence of the free fatty acid. These data demonstrate the relatively nonspecific nature of fatty acid stimulation of free biotin uptake by *L. plantarum*.

Effect of synthetic detergents on free biotin uptake. To test further the nonspecificity of this stimulation, various anionic, cationic, and nonionic synthetic detergents were examined for their effects on free biotin uptake.

Stimulation of vitamin uptake by the anionic detergent Aerosol OT (Fig. 3B) was slightly greater than the most effective concentration of oleic acid plus Tween 40 (Fig. 1B). These experi-

TABLE 1. *Effect of various agents on the uptake of free biotin*[a]

Expt	Agent tested	Reaction time	Concn tested (mg/ml)			
			0	0.1	1.0	25
		min				
1	Tween 20	7	140	165	174	178
	Tween 40			146	151	163
	Tween 60			151	155	144
	Tween 80			137	154	142
	Tween 20	30	253	324	335	251
	Tween 40			336	323	317
	Tween 60			279	340	330
	Tween 80			320	335	331
	Tween 80	60	278	326	394	260

Expt	Agent tested	Reaction time	Concn tested (μM)				
			0	50	100	200	400
2	CTAB	8	272	362	457	462	9
		16	667	825	930	445	7
		32	801	914	733	717	3

Expt	Agent tested	Reaction time	Concn tested (μg/ml)				
			0	10	50	100	200
3	Triton X-100	10	238	232	299	322	322
	Non-Ion-Ox			236	261	281	338
	Haemo-Sol				226	213	213
	Triton X-100	30	397	516	724	683	396
	Non-Ion-Ox			685	580	580	692
	Haemo-Sol			516	652	652	678
	Triton X-100	60	452	606	613	630	338
	Haemo-Sol				716	364	

Expt	Agent tested	Reaction time	Concn tested (μg/ml)				
			0	5	50	200	1000
4	DMSO	7	140	137	150	147	147
		30	253	244	282	244	248

Expt	Agent tested	Reaction time	Concn tested (M × 10^{-4})				
			0	0.1	0.5	1	5
	EDTA	7	140	128	125	121	125
		30	253	230	235	235	205

[a] Results are expressed as micrograms (× 10^{-4}) of biotin per milligram of dry cells at the various concentrations of test agents. Endogenous free biotin in micrograms (× 10^{-4}) of biotin per milligram of cells (dry weight): experiment 1 = 20; experiment 2 = 23; experiment 3 = 26; experiment 4 = 20.

ments were performed with the same crop of cells; hence, the comparison is valid. Since considerable variation in maximal levels of accumulated free biotin existed from experiment to experiment, such comparisons could be made only within a series of experiments employing a single crop of cells. As with free oleic acid, increasing concentrations of the detergent caused a rapid decrease in viability above 200 μmoles per ml (Fig. 3A) which was paralleled by a similar drop in biotin uptake (Fig. 3B). It may be significant that the point of maximal uptake occurred at the minimal concentration of detergent required to decrease viability (200 μM, 30 min). Experiments with SDS produced results identical to those shown for Aerosol OT. The cationic detergent CTAB exhibited patterns of stimulation and inhibition (Table 1, experiment 2) nearly identical to those observed with oleic acid (Fig. 1), Aerosol OT (Fig. 3), and SDS, except that peak activity and viability decreases occurred at lower concentrations (50 to 100 μM). This was expected since CTAB, and cationic detergents in general, are known to be more toxic to bacterial cells than are anionic detergents.

Nonionic detergents (Triton X-100 and Non-Ion-Ox) and the well-known laboratory detergent Haemo-Sol elicited responses identical with those previously described for oleic acid and various other detergents (Table 1, experiment 3). In all cases, the patterns of stimulation and inhibition of biotin uptake as well as the effects on cell viability were remarkably similar, further demonstrating a lack of specificity. The only exception to this pattern was a lack of Tween toxicity for the bacterial cells. This, however, was

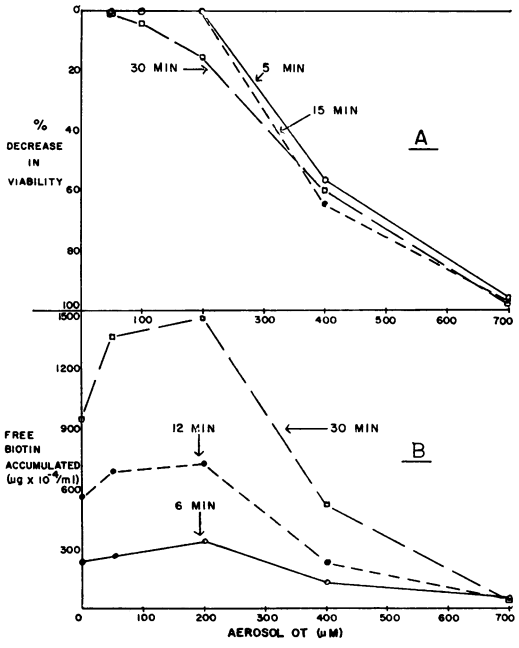

FIG. 3. *Effect of Aerosol OT on free biotin uptake and cell viability.*

expected, since these agents are known to be relatively nontoxic even in high concentrations.

Two other agents, dimethyl sulfoxide (DMSO) and ethylenediaminetetraacetic acid (EDTA), were tested for their ability to affect biotin uptake (Table 1, experiment 4). DMSO was selected because of its demonstrated rapid penetration through human skin. EDTA has recently been shown to cause nonspecific permeability increases in *Escherichia coli* (10). Neither of these agents affected free biotin uptake significantly.

DISCUSSION

These studies have shown that low concentrations of oleic acid and a variety of surface-active agents cause a marked stimulation of biotin uptake by cells of *L. plantarum*, whereas higher concentrations drastically reduce both cell viability and vitamin uptake. Fatty acids have surface-active properties and are known to interfere with microbiological assays for other vitamins, notably pantothenic acid and riboflavine, as well as biotin, when lactic acid bacteria are used. These results may, therefore, help to explain the relatively nonspecific effect of fatty acids on the growth of these organisms.

Williams, Broquist, and Snell (16) suggested that biotin is involved in the synthesis of oleic acid and that, when the latter is supplied preformed, biotin becomes nonessential. In contrast to these earlier studies, Broquist and Snell (6) later showed that avidin could eliminate completely all response to biotin even in the presence of oleic acid if the inoculum cells were derived from low biotin media. Moreover, they demonstrated that biotin absorbed by the inoculum cells could be used to support growth in the presence of oleic acid and avidin. They concluded that oleic acid reduced the biotin requirement of *L. plantarum* (formerly *L. arabinosus*) to a level which could not be observed directly and that this requirement could be supplied by synthesis when avidin was absent from the medium. However, since avidin does not penetrate bacterial cells, utilization of synthesized vitamin should not depend upon the absence of avidin, but should occur in its presence. This, in turn, should result in significant growth of the organism even in the presence of avidin. Thus, it appears quite certain that *L. plantarum* has an absolute requirement for biotin, supplied either endogenously as the result of accumulation (11, 15) or exogenously in the medium.

Based on experiments showing that oleic acid would not stimulate growth of *L. plantarum* in the complete absence of biotin, and that oleate inhibited the malic enzyme system only in intact cells, Traub and Lichstein (14) suggested that low concentrations of oleic acid and other fatty acids may exert their sparing effect by increasing cellular permeability to essential nutrients. They suggested further that higher concentrations of oleate may alter cellular permeability in a manner which would cause a loss of essential cell constituents or a decrease in the rate of entry of essential compounds into the cell, thus resulting in growth inhibition. Coles and Lichstein (7) showed that oleate inhibition of malic enzyme was due to leakage of the enzyme-nicotinamide adenine dinucleotide complex from the bacterial cells followed by dissociation of the complex. It was also shown that Tween 40 or lecithin detoxified the fatty acid (by acting directly upon the fatty acid rather than on the bacterial cells) and prevented inhibition of malic enzyme activity in whole cells. Our results and those of several investigators concerning the dual effects of surface-active agents on metabolism and viability of microorganisms (2, 3, 5) support this hypothesis, and again stress the nonspecificity of surfactants on cellular permeability. Also, Alexander and Trim (1) demonstrated that the penetration of hexylresorcinol into *Ascaris lumbricoides* was accelerated markedly by low concentrations of sodium cholate, sodium oleate, and CTAB, whereas high concentrations were completely inhibitory to such penetration.

During studies on lipid stimulation of *L. casei*, Williams and Fieger (18) reported that numerous synthetic detergents stimulated the response of this organism to biotin. They concluded that biotin functioned as a cell permeability factor and could be replaced by the proper lipids. Their data agree with the hypothesis of Traub and Lichstein (14) equally well, since it is possible that the detergents tested were acting as surface-active agents, increasing permeability and enabling the cells to utilize small quantities of the vitamin present in the medium and in the inoculum cells.

Surface-active agents are thought to exert their effects by combining with phospholipid components of the cell membrane, resulting in disorganization and disruption of its permeability properties (9). The mechanism by which these agents exert their stimulatory effects is not known. However, it may be that configurational changes in the enclosing layers of the cell could render transport systems more accessible to nutrilite molecules. It is also possible that the simple wetting action of surfactants could induce better contact between the cell and the surrounding medium, resulting in easier and perhaps more rapid uptake of essential components.

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